Production of Xylanase by Trichoderma sp. Via Solid State Culture Using Sugarcane Bagasse

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Abstract

Xylanase (E.C 3.2.1.8) is a wide spread group of enzyme which can catalyse the endohydrolysis of 1,4-β-D-xylosidic linkages in xylan. It has a wide range of application in industrial areas. Trichoderma sp. was selected as a potential producer of xylanase via a solid state fermentation system (SSF) using sugarcane bagasse as substrate. Fermentation was proceeded in the condition of 5.6 g sugarcane bagasse, 1% sucrose (w/w), 1% yeast extract as solid substrate and 70% moisture content (v/w). The aim of this study was to identify the ideal physical conditions of SSF for the highest xylanase acitivity. Thus, different fermentation days, concentration of carbon sources, moisture content and pre-incubation temperatures to the medium were conducted. The results obtained show that the higher xylanase enzyme activity of 380 U/g substrate DW was the maximum activity by using 1% sucrose as carbon source, 50oC of pre-incubation temperature, 70% (v/w) of moisture content at the fermentation day of six.

Keywords

Xylanase; Solid State Fermentation; Trichoderma sp. and Sugarcane Bagasse

Introduction

In solid state fermentation (SSF), xylanase is obtained as secondary metabolite from fungi cultivated on the lignocellulosic material as substrate. Lignocellulosic material can be found in most agricultural wastes, and is composed of cellulose, hemicellulose and lignin. Hemicelluloses are heterogeneous polymers of pentose, hexoses, and sugar acids. Hemicellulose, which is one part of the lignocellulosic, is the second most common polysaccharides. It represents approximately 20-35% of lignocellulosic biomass and the most abundant hemicellulose is known as xylan. Hence, xylanase can be found mostly in hemicellulose as it contains xylan. Agricultural wastes which consist of lignocellulosic material are the by-products of various agricultural activities, for example, crop production, crop harvest, saw milling and agro-industrial processing. Malaysia with abundant agricultural wastes will be of great advantage not only for the utilization of wastes for the production of value added chemicals but will also lessen the undesirable impact of the agricultural wastes on the environment. The major quantity of wastes generated from agricultural resources are palm kernel cake, sugarcane bagasse, paddy straw, rice husks and sago wastes, which contributes to more than 5 million tonnes of wastes per year (Pang & Ibrahim, 2005). Based on the huge amount of wastes, there is an urgent need to manage the bulk wastes effectively and economically. It is also necessary to generate value added products from these wastes (Pang & Ibrahim, 2005). This paper contributes to the production of xylanase using a sustainable raw material i.e. sugarcane bagasse as substrate which is content high carbohydrate, cheap and accessible to obtain throughout the year. Xylanase was produced using solid state fermentation which was found to be more economic due to the inexpensiveness and availability of agricultural wastes which can be used as substrate and can lead to the protection of environment (Slominski, Boros, Campbell, & Guenter, 2004).

Solid state fermentation (SSF) is generally defined as any fermentation process occurring in the absence or near-absence of free water, employing a natural

substrate or an inert substrate as solid support. In recent years, SSF has shown much progressive in the development of several biotechnology and products. Although a number of xylanase productions were performed using submerged systems but solid state fermentation was found to be more economical primarily owing to the low-cost and accessibility of agricultural wastes. The less stringent design requirements for such bioreactors and correspondingly lower costs could be considered as a favourable point for the SSF process providing an economic advantage over the submerged process (Pang & Ibrahim, 2005). The use of abundantly available and cost effective agricultural residues as substrates in SSF to achieve higher yield of products shows greater increment in the industrial sector. The major factors which affect microbial growth and activity in SSF include the selection of a suitable microorganism and substrates, moisture content, relative humidity, type and size of inoculums, period of cultivation and activity of the substrates (Beg, Kapoor, Mahajan, & Hoondal, 2001). Xylanase application can widely be found in the food and beverage industries, feedstock improvement and the quality improvement of lignocellulosic residues.

Lignocellulose is the main component of woody plants and non-woody plants (biomass) such as grass and represents a major source of renewable organic matter. Lignocellulose, which can be found also in sugarcane bagasse, consists of three main polymers which is cellulose, hemicellulose and lignin that are strongly intermeshed and chemically bonded by non-covalent forces and covalent cross linkages. The chemical properties of the lignocellulosic components make them a substrate of enormous biotechnological value (Howard & Jansen van Rensburg, 2003).

Enzymes are the catalytic cornerstone of metabolism that plays a central role in many manufacturing processes. Recently, many enzymes were produced via SSF such as xylanases, cellulases, lipases, mannanases, proteases, phytases and pectinases (Botella, Diaz, de Ory, Webb, & Blandino, 2007).

There are various group of microorganisms used in SSF for the production of metabolites specifically enzyme namely, bacteria, yeast and filamentous fungi such as *Trichoderma, Bacillus, Cryptococcus, Aspergillus, Penicillium, Fusarium, Humicola* and *Talaromyces* (Pang, Darah, Poppe, Szakacs, & Ibrahim, 2006). However, filamentous fungi are the most widely exploited because of their ability to grow on complex solid substrates and production of a wide range of extracellular enzymes. Extracellular enzyme is an enzyme that is secreted by a cell and that works outside of that cell. It is considered important from the industrial viewpoint as they simplify the extraction procedure (Pang et al., 2006).

Xylanases application can widely found in the food and beverage industries (clarification of juices and wines), feedstock improvement (improving the nutritional quality of silage and green feed), de-inking processes of waste papers and the quality improvement of lignocellulosic residues. It also permits bioconversion of lignocellulosic materials to produce fuel and other chemicals and with less disturbing of side reactions. Since the use of xylanase is beneficial for the society as well as for the environment (Ajay & Farhath, 2010), thus, the aim of this study was to identify the ideal physical conditions of SSF for the highest xylanase acitivity.

Materials and Methods

The sugarcane bagasse would be used as the substrate in the solid state fermentation. They were collected from freshly squeezed of sugarcane juice supplier, which is widely practised in Malaysia. The bagasse was washed thoroughly to make them dust-free. The sugarcane bagasse was milled using grinder and sieved between 1 to 3 mm of fibrous length. Then, the bagasse was dried at 100^{o}C for 24 hours. The strain of *Trichoderma* sp. was subculture on the Potato Dextrose Agar (PDA) plate and incubated for five days at 28^{o}C. After five days of growth of fungi, inoculums were prepared by scraping the sporangium from PDA and suspended in sterilized 25 mL distilled water for each petri dish. Then, the suspension of spores was filtered using Whatman filter paper no.1 and the filtrate was used as inoculum. Four petri dishes would be needed to make up 100 mL of spore suspension which then would be kept at 4^{o}C in chiller (Alam, Muhammad, & Mahmat, 2005). The cultivation of fungus was performed in 250 Erlenmeyer flask containing 5.6 g of sugarcane bagasse with addition of 2.0 mL of mineral salt solution. The mineral solution that prepared was composed of (in g/L) $MgSO_4.7H_2O$ 0.25, $(NH_4)_2SO_4$ 0.25, KH_2PO_4 0.25 and $ZnSO_4.7H_2O$ 0.005. Distilled water, mineral solution and inoculum were added to the growth medium to achieve 70% moisture content of the final substrate. The solid medium and the mineral solution have been autoclaved separately. After it was

autoclaved and cooled, the flasks were inoculated with 1 mL inoculums of *Trichoderma* sp and incubated at 30°C (Ikasari & Mitchell, 1996). The fermentation conditions were evaluated using different parameters such as fermentation days, moisture content, carbon source and pre-incubation temperature.

The fermented substrate was extracted with 70 mL of cold distilled water. The mixture was vigorously homogenized for 30 minutes at 200 rpm and the solid biomass residues were separated from the suspension by filtration through Whatman no.1 filter paper. The cell free supernatant was used as the source of the crude enzyme preparation (Pang & Ibrahim, 2005).

Xylanase activity was assayed and incubated in water bath at 50°C, in 0.05 M of citrate buffer at pH 5.3 for 20 minutes. Enzyme yield was expressed as U/g of dry bagasse. Xylanase activity was determined using 1% of xylan birchwood functioned as substrate in the enzyme reaction. The reducing sugar was determined by dinitrosalicylic acid method (DNS method) with D-xylose as standard reference at 540nm through spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme which releases 1 μmol of D-xylose in 1 minute under the assay condition (Maciel et al., 2008).

Results and Discussion

The optimization of fermentation process was carried out based on the stepwise modifications of the governing parameters for xylanase production. The traditional method of optimization which is 'one factor at a time' technique was used in this study. This technique indicates varying one factor while keeping the other factors at constant level (Alam et al., 2005).

Results of xylanase production are based on four parameters which are firstly, different fermentation days. The effect of fermentation day was examined from the first day until the seventh day in order to determine at which day the yield of xylanase was the highest. The next parameter to be studied was to determine the effect of supplementation of additional carbon source which is sucrose with different concentration. Next is the determination of the optimum moisture content for the highest titre of xylanase production. Lastly, the effect of pre-incubation temperature (during enzyme assay) was studied using different temperatures.

The production of xylanase was maximum at the sixth day with an enzyme activity of 316.25 U/g substrate DW. The lowest enzyme activity was 11.33 U/g substrate DW, obtained on the first day of the fermentation process. The enzyme activity pattern obtained from the result in the Figure 1 shows a slight increase from the first day to the second day, which theoretically is known as lag phase. This is the stage where the cells were in a period of adaptation to the new environment. There was only minimal increase in cell density and this caused low xylanase titres. Then, the pattern changes with a gradual increase from the second day to the sixth day. This stage is known as exponential phase. At this stage, the cells have adjusted to their new environment and the cells were divided into at constant rate resulting in an exponential increase in the number of presented cells. On the sixth day to the seventh day, there was a gradual decline in the enzyme activity. Theoretically, this is the decline phase or also known as death phase where the rate of dying cells was greater than the rate of dividing cells. Apart from that the depletion of one or more essential growth nutrients and accumulation of toxic growth associated by-product could be the reasons of the death phase. The finding obtained in this research which was the sixth day was the optimum day, inline with a study completed by Pal and Khanum (Ajay & Farhath, 2010). The xylanase production in their study was the highest on the day 6. On top of that, Okafor *et al*., (Okafor, Emezue, Okochi, Onyegeme-Okerenta, & Nwodo-Chinedu, 2007) also mentioned that the xylanase production using sugarcane bagasse as substrate by *Penicillium chrysogenum* (PCL501) shows the highest enzyme activity at day of six which is 1.39 U/mL^{-1}. Nevertheless, the enzyme activity obtained in this research was more than this previous research, due to the usage of different microorganisms. It is well known that *Aspergilus nige*r and *Trichoderma* are the big contributor in producing xylanase in the industries (Georgi, Ivam, Veselin, & Rositza, 2007). However, (Pang & Ibrahim, 2005) reported that the xylanase yield was the highest on the fifth day which is still considered not a big difference compared to day 6. Figure 1 shows a quite similar with the growth curve theoretically. According to Slominski *et al*. (Slominski et al., 2004), the decrease of the enzyme activity after the optimum temperature was due to the nutrients limitations and the nutrients may have been depleted due to the enzyme activity for the growth purpose.

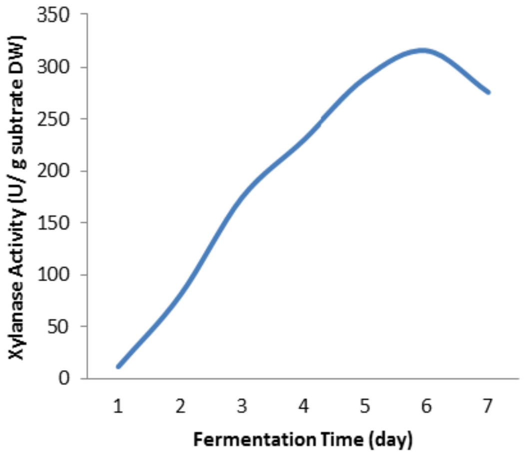

FIG. 1 XYLANASE ENZYME ACTIVITY AT DIFFERENT FERMENTATION TIME

Water is very important in solid state fermentation because solid substrates are non-soluble in water. The available water can absorb substrate particles which can be used by the microorganisms for growth and metabolic activity (Ashok Pandey, Carlos R. Soccol, & Mitchell, 2000). The degree of hydration of the substrate plays an important role in the growth of the fungi and subsequently the enzyme production. The moisture content was adjusted by adding the moistening agent to give the moisture content ranging from 50%, 60%, 70% and 80%. Figure 2 shows the effect of different moisture contents on the enzyme activity and xylanase production was optimum using sugarcane bagasse at 70% of moisture content which was 337.68 U/g substrate DW. The lowest enzyme activity was at the 50% of moisture content which was 108.65 U/g substrate DW. The enzyme activity pattern obtained shows the increment of xylanase enzyme activity as the moisture content increased but there was a gradual decrement of the xylanase enzyme activity at 80% of moisture content.

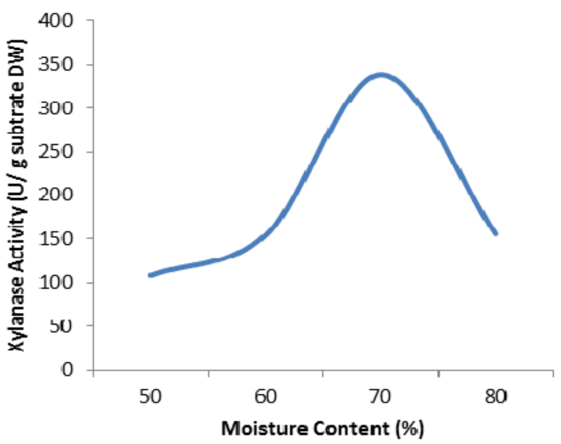

FIG.2 XYLANASE ENZYME ACTIVITY AT DIFFERENT MOISTURE CONTENTS

The xylanase enzyme activity was low at 50% because low water content is related to insufficient substrate swelling which prevented the nutrient absorption from the substrates. Furthermore, the lower moisture content leads to reduced solubility of the nutrients of the solid substrate, lower degree of swelling and a higher water tension (Ikasari & Mitchell, 1996). Besides that, 80% moisture content shows decrement in enzyme activity as the higher water content resulted in reduction in substrate porosity and caused oxygen limitation within the substrates which subsequently affected the oxygen transfer within the substrates thus resulting in poor growth (Rezende, Barbosa, Vasconcelos, & Endo, 2002).

Research done by (Pang et al., 2006), indicated that the production of xylanase enzyme using palm kernel cake was optimum at 75% of moisture content which was 19.5 U/g substrate DW. The enzyme activity of the previous research was very low compared to this research in which the highest enzyme activity of 337.68 U/g substrate DW was produced at 70% of moisture content. This is due to the effect of different agro waste used as the substrates. Different agro wastes used will contribute different characteristics of lignocellulosic materials such as lignin content, hemicellulose content (most of them consist of xylan), cellulose content, moisture content, initial pH and ash content. In another research done by Botella *et al*. (Botella et al., 2007), it was stated that the optimum xylanase production by *Aspergillus* sp. when sugar cane bagasse used as the substrate was at 70% of moisture content. Abdeshahian *et al*. (Abdeshahian, T., & Wan Yusoff, 2009) also highlighted that in his research, with using palm kernel cake and *Aspergillus niger* FTCC 5003, the highest enzyme activity obtained was at 63% of the moisture content.

As shown by Figure 2, the effect of moisture content indicates that the xylanase production was optimum using sugarcane bagasse at 70% of moisture content which is 337.68 U/g substrate DW. The lowest enzyme activity is at the 50% of moisture content which is 108.65 U/g substrate DW. Enzyme activity is low at 50% due to insufficient substrate swelling. The highest moisture content, 80% shows the lowest enzyme activity due to reduction in substrate porosity and leads to oxygen limitation and affects the oxygen transfer.

The production of primary metabolites by microorganisms is highly influenced by their growth, which is determined by the availability of the nutrients

in the substrates (Raimbault & Alazard, 1980). Therefore it is expected that the improvement of the nutritional value of the fermentation by the supplementation of carbon will also improve the enzyme production. Thus, the effect of carbon sources on the production of xylanase, besides the sugarcane bagasse (as prime carbon source) was tested using different concentration of sucrose. The enzyme activity of xylanase at different concentrations of sucrose which were (1, 2, 3, 4) % was depicted more clearly in Figure 3.

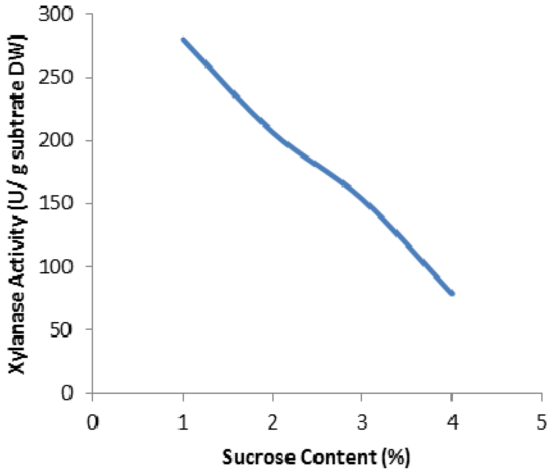

FIG.3 XYLANASE ENZYME ACTIVITY FOR DIFFERENT CARBON CONCENTRATION.

Sucrose is a disaccharide that linked two monomers, is rather complex, and the end result in each case is the loss of a hydrogen atom (H) from one of the monosaccharide and hydroxyl groups (OH) from the other. The highest enzyme activity was achieved when 1% of sucrose was used as the carbon source which is 280.36 U/g substrate DW, while the lowest enzyme activity was observed when 4% of sucrose was used as the carbon source which is 78.74 U/g substrate DW. Figure 3 shows the negative linear pattern of sucrose concentration versus xylanase enzyme activity. As the concentration of sucrose increases, the enzyme activity of xylanase decreases.

(Pang & Ibrahim, 2005) observed that the optimum carbon concentration was obtained when using 1% in the production of xylanase using palm kernel cake by *Aspergillus niger* USM AI 1 which is similar as in this study shown in Figure 3. On top of that, a previous study by (Pang et al., 2006) showed that maltose obtained maximum productivity of 28.80 U/mg glucosamine, however this study showed that enzyme activity production was at its highest at 1% of sucrose. This finding is in agreement with the report of Haltrich *et al*. (Dietmat Haltrich, Bernd Nidetzkym, KLaus D, And, & Zupancic, 1996).

Pre-incubation temperature is very important in determining the highest production of xylanase enzyme activity. Pre-incubation refers to the process of incubating 0.5 mL enzyme sample and 1 mL of birchwood xylan with 0.05 M citrate buffer (pH 5.3) in water bath for 20 minutes. This pre-incubation process will release the reducing sugar which will be measured using 3, 5-dinitrosalicylic (DNS) acid method described by Miller (Miller, 1959) and D-xylose as standard reference. The amount of releasing sugar is highly influenced by the temperature of the pre-incubation process of the enzyme. The higher the amount of the reducing sugar was released, the greater the xylanase enzyme activity was produced. Various temperatures (35, 40, 45, 50, 55)^{o}C for the pre-incubation of enzyme were studied in this experiment to determine the optimum temperature for the highest enzyme activity. Figure 4 shows the effect of the various pre-incubation temperatures in producing xylanase and the highest enzyme activity of xylanase was obtained at 50^{o}C of the pre-incubation temperature which is 380.01 U/g substrate DW. The lowest enzyme activity occurred when the temperature was set at 55^{o}C which showed the enzyme activity of 146.80 U/g substrate DW. As indicated in Figure 4, the enzyme activity kept on increasing as the temperature increased until it reached 50^{o}C and there is a gradual decrement after 50^{o}C of the pre-incubation temperature.

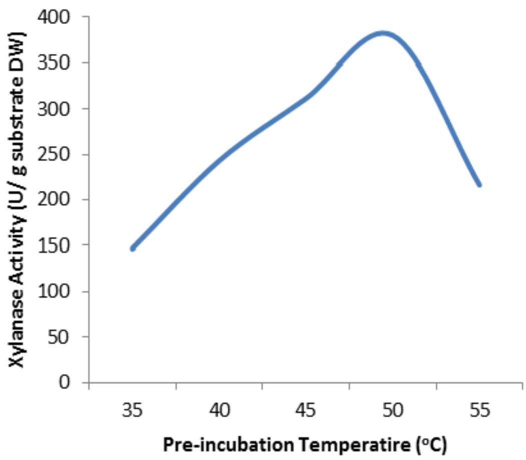

FIG.4 XYLANASE ENZYME ACTIVITY AT DIFFERENT PRE-INCUBATION TEMPERATURES.

Although the physiological changes induced by high temperatures during enzyme production are not completely understood, it has been suggested that at high temperatures, microorganisms synthesis only a

reduced number of proteins essential for growth and other physiological processes (Gawande & Kamat, 1998). With prolonged incubation, enzyme activity decreased sharply suggesting that the end-point of fermentation should be carefully controlled because synthesized xylanase could be degraded by non-specific proteases secreted by the fungus (Ajay & Farhath, 2010).

Conclusions

The fermentation conditions have been investigated aiming to achieve the highest xylanase activity. The constant parameters used were 10g of fermentation weight and 1% of yeast. It was discovered that the combination of 70% of moisture content at the sixth day, supplemented with 1% of sucrose and at 50^{o}C pre-incubation produced 380.01 U/g subtrate DW of xylanase activity.

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